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No deleterious mutations in the FOXJ1 (alias HFH-4) gene in patients with Primary Ciliary Dyskinesia (PCD)

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Abstract. The transcription factor FOXJ1 (alias HFH-4 or FKHL13) of the winged-helix/forkhead family is expressed in cells with cilia or flagella, and seems to be involved in the regulation of axonemal structural proteins. The knockout mouse *Foxj1*$^{-/-}$ shows abnormalities of organ situs, consistent with random determination of left-right asymmetry, and a complete absence of cilia. The human FOXJ1 gene which maps to chromosome 17q, is thus an excellent candidate gene for Kartagener Syndrome (KS), a subphenotype of Primary Ciliary Dyskinesia (PCD), characterized by bronchiectasis, chronic sinusitis and *situs inversus*. We have collected samples from 61 PCD families, in 31 of which there are at least two affected individuals. Two families with complete aciliogenesis, and six families, in which the affected members have microsatellite alleles concordant for a locus on distal chromosome 17q, were screened for mutations in the two exons and intron-exon junctions of the FOXJ1 gene. No sequence abnormalities were observed in the DNAs of the affected individuals of the selected families. These results demonstrate that the FOXJ1 gene is not responsible for the PCD/KS phenotype in the families examined.

The human homologue of the mouse hepatocyte nuclear factor 3/fork head homologue-4 (HFH-4) gene (also called FKHL13, forkhead-like 13, and recently renamed FOXJ1, Kaestner et al., 2000) was assigned to chromosome 17q22→q25 by homology with the mouse gene (Avraham et al., 1995) and mapped to chromosome 17q23→qter by human-rodent somatic cell hybrids (Pelletier et al., 1998).

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The human 2.5-kb FOXJ1 transcript encodes a 421-amino acid protein, which has a conserved DNA binding region of approximately 110 amino acids, characteristic of the winged helix family of transcription factors. The gene spans approximately 6 kb and contains only one intron which divides the coding region of the forkhead DNA binding domain (Fig. 1) (Murphy et al., 1997; Pelletier et al., 1998).

The expression of FOXJ1 has been studied in both mice and humans. The FOXJ1 protein has been localized in the nuclei of various tissues including developing and adult proximal respiratory airways (nose, paranasal sinuses, trachea, bronchi, and bronchioles), choroid plexus, ependymal cells, seminiferous tubules of the testis, oviduct, human fetal kidney and mouse fetal esophageal epithelium (Hackett et al., 1995; Murphy et al., 1997; Lim et al., 1997; Pelletier et al., 1998; Blatt et al., 1999; Tichelaar et al., 1999). In the developing mouse and human lung, FOXJ1 expression is restricted to the proximal respiratory epithelium and it is associated with the differentiation of proximal from distal pulmonary epithelium. Moreover,

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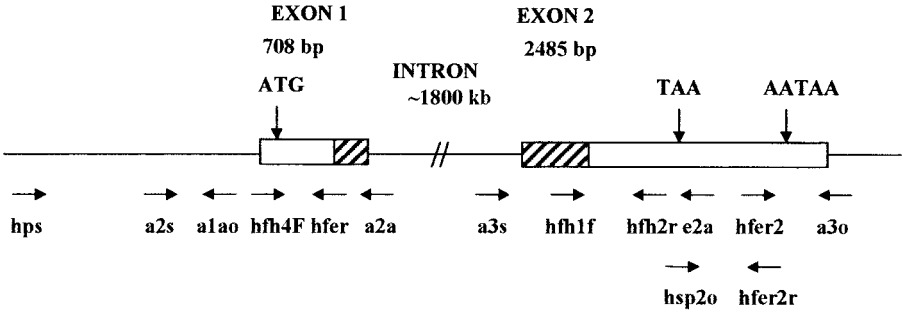

Fig. 1. The FOXJ1 gene is composed of two exons (white boxes) with the DNA-binding domain (shaded boxes) split between exons 1 and 2. Horizontal arrows show the position and the orientation of PCR and sequencing primers used in this study.

FOXJ1 is expressed in respiratory cells with motile-type cilia, but not in those with sensory cilia. The pattern of expression restricted to ciliated epithelial cells, and the temporal relationship of FOXJ1 expression to the development of cilia and flagella is consistent with a role for FOXJ1 in the differentiation and maintenance of the ciliated cell (Blatt et al., 1999).

The mouse with targeted disruption of the *Foxj1* gene exhibits prenatal and postnatal growth failure, perinatal lethality, infertility and, in some cases, hydrocephalus (Chen et al., 1998). Homozygous *Foxj1*$^{-/-}$ mutant mice revealed heterotaxy (with either reversal of the abdominal viscera or dextrocardia) or situs inversus totalis in 50% of the animals, consistent with random determination of left-right asymmetry. Moreover, these mice showed a complete absence of cilia, or flagella in sperm (Chen et al., 1998). In addition, there is absence of left-right dynein (lrd) expression in the embryonic lungs, suggesting that Foxj1 may act by regulating expression of dyneins (Chen et al., 1998). FOXJ1 is, therefore, an outstanding candidate gene for Primary Ciliary Dyskinesia (PCD) and Kartagener Syndrome (KS).

In KS (OMIM 244400), a subphenotype of PCD (OMIM 242650), abnormalities of organ situs are associated with defects in the development of cilia. PCD (also known as Immotile Cilia Syndrome, ICS) is a genetic disorder, usually inherited in an autosomal recessive mode. Ciliary motility is affected, with a severity ranging from dysmotility to complete immobility of cilia and flagella. This results in multisystemic diseases of varying severity and penetrance, characterized by bronchiectasis, chronic sinusitis and male infertility. Half of the patients show dextrocardia, with or without situs inversus totalis. KS is characterized by the triad of bronchiectasis, chronic sinusitis and situs inversus. Ultrastructural defects of cilia involving dynein arms are frequent in PCD patients (Afzelius and Mossberg, 1995). Genetic heterogeneity, expected because of the complex architecture of cilia, has been demonstrated by linkage studies (Blouin et al., 2000; Meeks et al., 2000). Recently, mutations in the DNAI1 gene, encoding a member of the intermediate dyneins, have been demonstrated in a single patient with PCD (Pennarun et al., 1999).

We undertook mutation analysis in the FOXJ1 gene in several pedigrees with PCD and failed to detect a deleterious mutation. We conclude that FOXJ1 is not involved in the PCD phenotypes in the families studied.

Table 1. Sequences of the primers used in this study. Their orientation and position within the FOXJ1 gene are shown in Fig. 1.

Primer	Sequence
a1ao	GAAACAGAAGCAAGGCCCTG
a2a	AGAGGGAGGCAGATCTGATG
a2s	ATATGAATCCAGTGCACCGT
a3o	GGGCTCAGTGTTCTGGAGA
a3s	ACTGACCTAGCGGTTCCTCT
e2a	GCTGGTGGCCATGTGGCCTT
Hfer	GAATGGAGAATTCCTGCAGC
hfer2	TGTAGCTGAGGCTTAACTGG
hfer2r	CAGTTAAGCCTCAGCTACAGC
hfh1f	CTGACGGTGAATACCGAGG
hfh2r	CTTGGGCAGAGGGTGTTTGG
hfh4F	GACTCAGCGGGCCAGA
Hps	GGGACTTAGCGGCGGGTAG
hps2o	TTCTTGTAAGAGGCCAGGCC

Materials and methods

Clinical samples, family selection

As part of an international collaboration, we collected more than sixty well-characterized families with PCD, 31 of which have at least two affecteds. A genome-wide scan was performed on the 31 multiplex families, and the results showed extensive genetic heterogeneity (Blouin et al., 2000). We have selected samples from eight families to perform mutation analysis of the FOXJ1 candidate gene. Six families were selected because the patients per family shared the same maternal and paternal haplotypes for chromosome 17q23→qter markers. The genotypes were determined as previously described (Blouin et al., 2000). Two further families were selected because ultrastructural analysis of nasal epithelium showed complete aciliogenesis, a phenotype similar to that of the *Foxj1*$^{-/-}$ mouse.

Mutation search

The Genbank database contains two different FOXJ1/HFH-4/FKHL13 sequences: U69537 (cDNA, Pelletier et al., 1998) and X99349/X99350 (genomic) and NM_001454 (cDNA, Murphy et al., 1997). We used these sequences to define oligonucleotide primers as shown in Fig. 1 and Table 1.

The first exon was amplified with primers a2s and a2a (Table 1). 30 cycles of PCR amplification were performed with denaturing at 94°C for 1 min, annealing at 51°C for 1 min, and elongation at 72°C for 2 min, in the presence of 12% DMSO. The 909-bp product was directly sequenced with primers a2s, hfh4f and hfer.

The coding region of the second exon was amplified with primers a3s and e2a. The amplification was performed with an annealing temperature of 52°C and 14% DMSO, and resulted in a 1,017-bp product which was directly sequenced with primers e2a, hfh1f and hfh2r.

The 3′ end of the gene, containing the poly(A) signal consensus, was amplified in two rounds with primers hps2o and hfer2r and primers hfer2 and a3o at an annealing temperature of 50°C and 10% DMSO. The 805 and

Table 2. List of sequencing variants (polymorphisms and potential previous sequencing errors) found when comparing the sequences obtained in this study with the two published sequences of FOXJ1 (Murphy et al., 1997; Pelletier et al., 1998). They are divided into 5′UTR, coding region of exon 1, coding region of exon 2 and 3′UTR. [a] The nucleotide positions correspond to sequence AJ272064.

5' UTR

Position[a]	15	150	185-189	226-227
Murphy et al	CCACCT	AGAAT	AGTCTG_GG	CCC___GG
Pelletier et al.				CCCCCGG
This work	CCAGCT	AG_AT	AG_CTGCGG	CCCCCGG

Exon 1

Position[a]	290	306	311	324	329	339	442-443
Murphy et al.	TGTCTG	GGAACCGGCCCG		GAG_CCGG_CCGGAGGGC_GCCTG			GGCACC
Pelletier et al.	TGGCTG	GGAGCCGGGCCG		GAGGCCGGGCCGGAGGGCGGCCTG			GCGACC
This work	TGGCTG	GGAGCCGGGCCG		GAGGCCGGGCCGGAGGGCGGCCTG			GGCACC
Amino acid sequence	W L	G A G P		E A G P E G G L			G T
Amino acid change	yes	yes	no	Reading frame disrupted and restored			yes

Exon 2 (coding region)

Position[a]	1044	1060	1066-1068	1088	1096	1103	1106	1116	1136	1205-1206	1263
Murphy et al.	GAGCCC	GCGGTCTGGG_TACAGGCGAGGGCAGGCTGGGACATAAGCCCAAACACCCTCTGCCCAAG_GGGTGGCCAAGGTCCCGCG_CCCCCC								GACTGG	GCCCTG
Pelletier et al.	GAGGCC	GCGGGCTGGGGTGCAGGCGAGGGCAGGCTGGGGCATAAGCGCAAACAGCCGCTGCCCAAGCGGGTGGCCAAGGTCCCGCGGCCCCCC								GACCTG	GCCGTG
This work	GAGGCC	GCGGGCTGGGGTGCAGGCGAGGGCAGGCTGGGGCATAAGCGCAAACAGCCGCTGCCCAAGCGGGTGGCCAAGGTCCCGCGGCCCCCC								GACTGG	GCCCTG
Amino acid sequence	E A	A G W G A G E G R L G H K R K Q P L P K R V A K V P R P P								D W	A L
Amino acid change	yes	reading frame disrupted and restored								yes	yes

3' UTR

Position[a]	1546	1590	1632	1651	1666-1669	1672	1681
Murphy et al.	GAGGCCAG	TCCAAAACT	CCTATGCA	GGGCTCCAA	CCCCAAAGGCCACATGGCCACCAG		
Pelletier et al.	GAGGTCAG	TCCAGAACT	CCTAGGCA	GGG_TCCAA	CCCAG_AGG_CACATGGC_ACCAG		
This work	GAGGCCAG	TCCAGAACT	CCTAGGCA	GGGCTCCAA	CCCCAAAGGCCACATGGCCACCAG		

the 299-bp products were sequenced with the same primers used for PCR amplification. A 787-bp long putative promoter region was amplified with primers hps and a1ao at an annealing temperature of 51 °C. Nucleotide sequencing was performed using the ABI377 automated sequencer; assembly and comparison of sequences were done using the gap4 program from the STADEN package (Staden, 1996).

Results and discussion

We have performed nucleotide sequencing of the coding region, the 5′ and 3′ UTRs of the FOXJ1 gene, from PCR amplified DNA of eight selected PCD patients and two heterozygous parents. This screening for mutations did not show any putative pathogenic mutation. Moreover no polymorphic variants were detected. There were, however, some differences between the sequences of this study and those previously deposited in the public databases. Some of these variations are probably polymorphisms, whereas others could be due to sequencing errors. A list of the differences observed is given in Table 2. The cDNA sequence from this study has been deposited in the public databases with accession number AJ272064.

We have searched for mutations in the human FOXJ1 gene in the DNAs of selected patients with Primary Ciliary Dyskinesia (PCD). The rational for this study was that mice with targeted disruption of the *Foxj1* gene lacked cilia and showed situs abnormalities. Furthermore, the expression of FOXJ1 protein suggests that it is involved in epithelial cell differentiation and ciliogenesis (Blatt et al., 1999). For the mutation search, we selected samples from six PCD families compatible with linkage to chromosome 17q23→qter where FOXJ1 maps, and two PCD families with absence of cilia in nasal epithelium. No mutations were found in the affected members of these families suggesting that the FOXJ1 gene is not responsible for the PCD phenotypes in these families. Although the exclusion of FOXJ1 as a candidate gene for PCD is based on the mutation search of the entire coding region, intron-exon junctions and the 5′ and 3′ UTRs, it is theoretically possible that mutations outside of these regions could be responsible for the PCD phenotype in some of these families.

PCD is thought to be a heterogeneous disorder due to mutations in several different genes. We recently performed a genome-wide linkage analysis in 31 multiplex families with

PCD in order to identify the genomic location of genes involved in this phenotype. No locus for the majority of the families was identified, although the sample was powerful enough to detect linkage if 40% of the families were linked to one locus. Potential genomic regions harboring PCD loci were localized on chromosomes 3p, 4q, 5p, 7p, 8q, 10p, 11q, 13q, 15q, 16p, 17q, and 19q (Blouin et al., 2000). Lod scores of 1.50 with markers on 17q were obtained using non-parametric linkage analysis in the subset of PCD families with either situs inversus or dynein arm deficiency.

Using a candidate gene approach, the first gene involved in PCD has been recently determined. Two loss-of-function mutations in the human DNAI1 gene on chromosome 9p21→p13 encoding a dynein intermediate chain (which is related to IC78 of the *C. reinhardtii* gene) have been found in a patient with PCD characterized by immotile respiratory cilia lacking outer dynein arms (Pennarun et al., 1999). It is likely that additional dynein genes are involved in the molecular pathophysiology of the disease.

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